

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.



⑫

## EUROPEAN PATENT APPLICATION

⑳ Application number : 92610073.6

⑤① Int. Cl.<sup>5</sup> : **C12Q 1/68, C12N 15/31,  
C07K 13/00, C07K 7/00,  
A61K 39/02**

㉔ Date of filing : 22.10.92

③① Priority : 22.10.91 US 779185

④③ Date of publication of application :  
05.05.93 Bulletin 93/18

⑧④ Designated Contracting States :  
PT

⑦① Applicant : SYMBICOM AB  
Tvistevägen 48, P.O.Box 1451  
S-901 24 Umea (SE)

⑦② Inventor : Barbour, Alan, George  
404 Charles Road  
San Antonio, Texas 78209 (us)  
Inventor : Bergström, Sven  
Marmorvägen 95  
S-902 40 Umeå (SE)

⑦④ Representative : Andersen, Henrik Rastrup et  
al  
c/o Plougmann & Vingtoft Sankt Annae Plads  
11 P.O. Box 3007  
DK-1021 Copenhagen K (DK)

⑤④ Improvements in *Borrelia burgdorferi* diagnosis and prophylaxis.

⑤⑦ Methods of diagnosing borreliosis, Lyme disease, by detecting the presence of a *Borrelia burgdorferi* organism in a specimen from an animal, including a human, by the use of PCR-DNA analysis, where the primers are subsequences from OspA and OspB DNA sequences from any of the three *B. burgdorferi* strains: B31, Ip90 (or Iper90) and ACAI. The method may be used for detecting the presence of *B. burgdorferi* in a specimen irrespective of the species and strain of the *B. burgdorferi* organism proposed to be in the specimen, but may also be used for detecting specific strains, depending on the primers chosen. Vaccines comprising polypeptides capable of evoking an immune response against OspA or OspB of *B. burgdorferi* organisms for active immunization. DNA sequences encoding the polypeptides.

EP 0 540 457 A1

The present invention relates to valuable and useful developments based upon new findings about DNA and peptide sequences from the Borrelia burgdorferi strains ACA1 and Ip90 and their relationship to Borrelia burgdorferi strain B31, including special sequences from the three different strains of Borrelia burgdorferi (from now on B. burgdorferi) useful as primers in the PCR-DNA detection of B. burgdorferi in specimens from animals, including humans, a diagnostic kit for diagnosing Lyme disease in animals, including humans, DNA sequences from B. burgdorferi strains ACA1 and Ip90 encoding polypeptides related to the outer membrane protein OspA and OspB, a polypeptide encoded from ACA1 and Ip90, epitopes from OspA and OspB, and a vaccine against Lyme disease comprising one or more epitopes from OspA and OspB.

## 10 BACKGROUND OF THE INVENTION

Lyme disease is a zoonosis caused by the tick-borne spirochaete B. burgdorferi. When a susceptible host is bitten by an ixodid tick, B. burgdorferi organisms enter the skin. In humans the initial skin manifestation is termed erythema chronicum migrans (ECM) whereas a long-standing infection of the skin produces acrodermatitis chronica atrophicans. The Borrelia organisms also enter the circulatory system of the host and are distributed to various organs, including the brain and joints. A secondary spread of the pathogens produces a variety of clinical syndromes, including lymphocytic meningoradiculitis, myocarditis and chronic arthritis. In many patients the infection of some tissues, particularly the brain and joints, persists for years and can be severely disabling. These forms of chronic Lyme disease are a consequence of the host's inability to rid itself of the infectious agent and perhaps the development of an autoimmune reaction.

Diagnosis of Lyme disease has chiefly been based on clinical evidence. The best marker during the primary stage of infection has conventionally been the presence of erythema chronicum migrans (ECM) but these skin lesions may not always develop or they may manifest atypically. Moreover, Lyme disease can be confused with other illnesses characterized by neurologic or arthritic manifestations. When clinical histories are incomplete, serologic testing with determination of antibody titers is the best conventionally laboratory method of diagnosis. Indirect fluorescent antibody (IFC) staining tests and enzyme-linked immunosorbent assays (ELISA) are used to detect total immunoglobulins or class-specific IgM and IgG antibodies to B. burgdorferi. ELISA is usually preferred because the procedures are more easily standardized and automated and because absorbance values can be statistically analyzed to give more objective results.

B. burgdorferi spirochaetes are helically shaped, motile cells with an outer cell membrane that surrounds a protoplasmic cylinder complex, consisting of the cytoplasm, the cell wall, the inner cell membrane and the flagella which are located not at the cell surface but in the periplasmic space between the outer cell membrane and the protoplasmic cylinder. The outer cell membrane and the flagella are assumed to play an important role in the host-parasite interactions during the disease and has been subjected to several investigations, identifying major surface-exposed proteins as important immunogens.

It has been shown that the earliest IgM antibodies formed against antigens of the B. burgdorferi strain B31, which was deposited in the American Type Culture Collection in 1983 with the accession number ATCC 35210, are directed against a genus-specific flagellar polypeptide termed flagellin having a molecular weight of 41 kd (18) and which reacts with monoclonal antibody H9724. IgG antibodies are also first directed to the 41 kd flagellin, but with advancing disease IgG antibodies form against other immunogens, especially against two abundant proteins with molecular weights of 31 kd and 34 kd. These two proteins, which have been denoted OspA (31 kd) and OspB (34 kd), have been found to be located at the B. burgdorferi surface and embedded in its outer fluid cell membrane. The OspA protein has been found to be less variable in its molecular weight and in its reactivity with monoclonal antibody H5332 (10), whereas the molecular weight of OspB proteins from different B. burgdorferi strains vary and the OspB proteins of different strains also show varying reactivity with two monoclonal antibodies against OspB (H6831 and H5TS) (9). The main variation among OspA proteins is found between isolates from Europe and the United States.

Conventional diagnostic tests for Lyme disease have used whole spirochaetal sonic extracts as test antigens in ELISA to detect antibodies to B. burgdorferi, but this test yields unsatisfactory low diagnostic sensitivity (20 to 60%) during the early stage of infection, possibly due to a slow and late-appearing antibody response and to the inclusion of irrelevant cross-reacting antigens in the whole-cell preparations. In addition, the use of whole cells as test antigens may result in the occurrence of false positive reactions. For example, among patients with syphilis and in areas where a closely related relapsing fever Borrelia spp. co-exist with B. burgdorferi, serologic differentiation of Lyme disease from tick-borne relapsing fever is difficult. Detection of IgG antibody to B. burgdorferi in later stages of infection can help in distinguishing Lyme disease from aseptic meningitis, multiple sclerosis, serum negative rheumatoid arthritis, juvenile rheumatoid arthritis, and Reiter's syndrome.

The antigen-antibody diagnostic approach has been found not to work as well in connection with Lyme

disease as in connection with many other infectious diseases.

One of the reasons for this is that only a low number of spirochaetes is present and that the antigens in the outer membrane of the spirochaetes are hard to detect for the immune system of the infected organism.

Another reason is that the antibody response to the B. burgdorferi infection first arises weeks after the bite of the tick, and in many cases first after the patient has shown clinical signs of the disease.

It would be desirable to provide a diagnostic tool which is able to diagnose a B. burgdorferi infection at all stages, also, at very early stages even before the clinical signs of infections appear and the diagnostic tool being independent of the causative infective B. burgdorferi strain.

A most promising and sensitive method for diagnosing the Lyme disease agent is that of detecting a single B. burgdorferi organism by PCR amplification.

Nielsen S.L. et al. Molecular and Cellular Probes (1990) 4, 73-79, Detection of Borrelia burgdorferi DNA by the polymerase chain reaction, and Malloy, D.C. et al, Journal of Clinical Microbiology, June 1990, p. 1089-1093, Detection of Borrelia burgdorferi Using the Polymerase Chain Reaction, both disclose the use of DNA-sequences from only B. burgdorferi strain B31 in the preparation of primers useful in the PCR-DNA diagnostic of Lyme disease.

WO 91/06676 discloses the use of DNA primers associated with the SC plasmid in the diagnostic of Lyme disease, but does not disclose the sequence of the primers.

EP 421 725 A1 discloses nucleic acid probes useful for identifying B. burgdorferi in samples. The probes are designed from B. burgdorferi strains in The United States and Europe.

EP 445 135 discloses the use of a DNA fragment from the OspA gene from the B31 strain in PCR-DNA diagnostic of B. burgdorferi infection in mammals, including humans, as well as the use of immunogenic polypeptides found to be antigenic when assessed with monoclonal antibodies directed against OspA in the preparation of a vaccine immunizing mammals, including humans, against Lyme disease. EP 445 135 discloses, inter alia, three contemplated epitopes which are small fragments of B31 OspA: Lys-Glu-Lys-Asn-Lys-Asp, Ser-Lys-Lys-Thr-Lys-Asp, and Lys-Ala-Asp-Lys-Ser-Lys. To the extent this is relevant, these fragments and their utility and use as epitopes is disclaimed in the present invention.

Rosa, P.A. et al., Journal of Clinical Microbiology, Mar. 1991, p.524-532, Polymerase Chain Reaction Analyses Identify Two Distinct Classes of Borrelia burgdorferi discloses PCR primers used in the identification.

One object of the present invention is to provide non-immunological assays by providing nucleotide sequences which can hybridize with different strains of B. burgdorferi from different geographical regions so that a diagnostic tool for detecting B. burgdorferi spirochaetes independent of causative infective strain of B. burgdorferi is obtained. Another object is to provide novel DNA fragments related to B. burgdorferi. A further object of the invention is to provide antigenic polypeptides, an antigenic composition and a vaccine for immunizing animals, including humans, against Lyme disease substantially independent of the infective B. burgdorferi strain causing the disease.

## DESCRIPTION OF THE INVENTION

In one aspect, the invention relates to a method for detecting the presence of a B. burgdorferi organism in animals, including humans, the method comprising subjecting a specimen from the animal, such as a body fluid, such as blood, serum, cerebrospinal fluid, synovial fluid, pericardial fluid or urine, or a tissue biopsy, or, when the animal is a anthropod, the whole animal, to PCR-DNA analysis which either

A) uses a nucleotide primer sequence which comprises at least 11 nucleotides, the primer sequence being identical or substantially identical to a sub-sequence of at least two of the three DNA sequences derived from B. burgdorferi strains B31, Ip90 and ACA1, respectively, shown in Fig. 3 herein,

B) uses a combination of at least two nucleotide primer sequences, each of which comprises at least 11 nucleotides, one sequence being identical or substantially identical to a sub-sequence from the OspA and OspB DNA from a first B. burgdorferi strain as shown in Fig. 3 herein, another sequence being identical or substantially identical to a sub-sequence from the OspA and OspB DNA from another of the B. burgdorferi strains as shown in Fig. 3 herein, and optionally a third sequence which is identical or substantially identical to a sub-sequence from the third of the B. burgdorferi strains as shown in Fig. 3 herein,

the substantial identity being such that the said primer sequence will hybridize to the sub-sequence under conventional hybridization conditions (reference Sam Brooks), or under high stringency hybridization conditions comprising hybridization at 67°C in 2xSSC and final washing at 67°C in 1xSSC, or under medium high stringency hybridization conditions comprising hybridization at 67°C in 3xSSC and final washing at 67°C in 1xSSC.

This method proves a diagnostic tool which may be used to diagnose B. burgdorferi infection at all stages, even before the patient shows any clinical signs of Lyme disease, because the B. burgdorferi spirochaetes are detected instead of the antibodies towards the spirochaetes. Also, the method is able to detect even a

single B. burgdorferi spirochaete and therefore the sensitivity of the method is very high.

Furthermore, the method is useful in diagnosing whether a Ixodes tick is a vector for B. burgdorferi, which is of especially interest in areas with endemic B. burgdorferi infections.

It has previously been shown that B. burgdorferi strains of different geographical origin differ in DNA sequence profiles, and until now two classes of B. burgdorferi have been disclosed (36).

According to the present invention the OspA and OspB genes from two B. burgdorferi strains ACA1 and Ip90 have been sequenced and compared to the OspA and OspB gene sequence from strain B31, previously sequenced (14). The differences seen when comparing the sequences from B. burgdorferi strains B31, ACA1 and Ip90 indicate that B. burgdorferi may be placed into three different classes, using B31, ACA1 and Ip90 as reference strains, instead of placing B. burgdorferi into two classes.

The B. burgdorferi strain ACA1 is a Swedish isolate from the tick vector I. ricinus, and the B. burgdorferi strain Ip90 is an isolate from the Soviet Union from a region in which the tick vector is I. persulcatus. The known B. burgdorferi strain B31 is a North American isolate from the tick vector I. dammini.

Comparison between the known B31 sequence and the two novel sequences from ACA1 and Ip90, respectively, reveals that there are several regions having conserved sequences either totally or with a few mismatches of nucleotides in the regions, Fig. 3.

As mentioned above, the strains discussed herein are from different geographical regions. According to one aspect of the invention, therefore, primers are provided which can detect B. burgdorferi of all classes by the PCR amplification method, by using nucleotide sequences from regions having the same or substantially the same DNA-sequence in all the three strains.

In the present context the term "substantially identity" is intended to indicate that the sequences only have one or a few mismatches, and that these mismatches will not interfere with the annealing of the primer sequence in question to its target sequence with sufficient specificity. A more precise definition is based on hybridization: A primer sequence which is substantially identical with a particular sub-sequence in the context of the present invention will hybridize to the sub-sequence under defined hybridization conditions. Several sets of conventional hybridization conditions relevant in the present context are described in (52). For a primer, however, a rather high degree of specificity is often required, and this is reflected in the above specification of high stringency hybridization conditions comprising hybridization at 67°C in 2xSSC and final washing at 67°C in 1xSSC, or medium high stringency hybridization conditions comprising hybridization at 67°C in 3xSSC and final washing at 67°C in 1xSSC.

The primers may, in principle, be shorter than the 11 nucleotides mentioned above, but this is normally not preferred for specificity reasons. On the contrary, it is normally preferred that the primers are somewhat longer, such as comprising at least 12 or 13 nucleotides, preferably at least 15 nucleotides, and in many cases about 18 nucleotides or even higher.

The most interesting primers for use in a general PCR-DNA detection of B. burgdorferi are, of course, primers which are especially capable of amplifying DNA from a B. burgdorferi irrespective of the particular strain of the B. burgdorferi. Primer sequences of particular interest in this connection are primers corresponding to sub-sequences which are identical or substantially identical in all three of the strains B31, Ip90 and ACA1. Primers fulfilling this condition are, e.g. primers which are fragments comprising a sequence which is identical or substantially identical to one of the following sequences (or one of their complementary sequences):

5' -GTATTAAGTTATATTAATAT-3'

5' -AAAAGGAGAATATATTATGA-3'

5' -AAAAATATTTATTGGGAATA-3'

5' -GGAAAAGCTAAAGAGGTTTTAAAA-3'

5' -ACTTCAACTTTAACAATTA-3'

5' -AATAAGGAGAATTTATGA-3'

5' -AAAAAACTAAA-3'.

As will be seen from Fig. 3 which shows the DNA sequences (a single strand of the double-stranded DNA shown) of the *B. burgdorferi* strains B31, ACA1, and Ip90, the above specific DNA fragments represent regions showing full or almost full identity between the three strains.

While PCR analysis can be performed using a single primer, it is, of course, normally more advantageous to utilize the exponential amplification obtained when using a set of primers amplifying complementary strands such as is normal in PCR. Therefore, in a preferred embodiment of this aspect of the invention, a set of primers is used, the first primer of the set being a primer as defined above, the second primer comprising a sequence which is identical or substantially identical to a subsequence of a strand complementary to OspA or OspB DNA from at least one of *B. burgdorferi* strains B31, Ip90 and ACA1 as shown in Fig. 3 herein, the subsequence of the complementary strand being downstream of the first primer in relation to the direction of expression of the first primer, the substantial identity being as defined above. When a set of primers is used, it is normally preferred that they have substantially the same degree of identity with their target sequences.

While the embodiments discussed above focus on the establishment of a PCR analysis which will detect DNA from a *B. burgdorferi* spirochaete irrespective of which strain it is, another interesting aspect of the invention is a PCR analysis making it possible to detect the presence of a *B. burgdorferi* spirochaete in a specimen with strain specificity, in other words to detect specifically a *B. burgdorferi* of one of the now-identified three main classes. In this aspect, the specimen is subjected to PCR-DNA analysis using as a primer a DNA sequence which comprises at least 11 nucleotides, the sequence being identical or substantially identical to a subsequence of OspA and OspB DNA from one of the *B. burgdorferi* strains B31, Ip90 and ACA1 as shown in Fig. 3, which subsequence is different from any subsequence of the two other strains, the difference being such that the sequence will not anneal to DNA from the two other strains, the substantial identity being as defined above. The difference in sequences which is decisive in this type of PCR according to the invention can be identified directly from Fig. 3 and confirmed by simple experiments.

Again, the length of the primer is preferably at least 12, more preferably at least 13 or 15 and often about 18, such as explained above, and also in this case, a set of primers is preferably used.

The PCR analysis using the special primers discussed herein is performed in accordance with usual PCR technique such as described in the literature, e.g. (51). Thus, for the detection, the primer may be labeled, such as with radioactive labels, fluorescent dyes, and biotin, or labeled nucleotide triphosphates (e.g. labeled with thymidine) can be included in the PCR reaction to label the PCR amplification product.

The PCR primers used according to the invention may be prepared using well-known methods. Thus, they may be prepared by oligonucleotide synthesis, or they may be prepared by fragmentation of a larger nucleotide sequence using suitable restriction enzymes. The labelling of the primers can be performed by methods well-known *per se*.

As is conventional, the PCR reagents can be included in suitable PCR kits.

While the above discussion emphasizes the use of a single kind of primer capable of detecting all the strains of *B. burgdorferi*, in a universal test, such a test can, of course, also be established by using a combination of different primers, comprising, for each of the strains of *B. burgdorferi* to be detected, a type of primer unique to the strain.

Using the PCR analysis method or kit as described above, it is possible to diagnose Lyme disease as well as pre-clinical borreliosis in mammals, including humans, independent of the strain of B. burgdorferi that have caused the disease.

In another embodiment, the present invention relates to important novel DNA fragments and their use.

5 DNA fragments of the invention are fragments encoding the OspA and OspB of B. burgdorferi of the Swedish strain ACA1 and of the Soviet strain Ip90 or any modifications of said sequence encoding a polypeptide which is functionally equivalent to OspA and OspB from ACA1 and Ip90, respectively.

The term "functional equivalent" is intended to include all immunogenically active substances with the ability of evoking an immune response in animals, including humans to which the equivalent polypeptide has been administered, e.g. as a constituent of a vaccine which immune response is similar to the immune response evoked by the OspA and OspB. Thus, equivalent polypeptides are polypeptides capable of conferring immunity to Lyme disease.

OspA and OspB are outer surface proteins in B. burgdorferi which are encoded by DNA-sequences found within the same operon. The genes encoding OspA and OspB are located on a double stranded linear plasmids.

15 One embodiment of the invention is a DNA fragment comprising a nucleotide sequence as shown in Fig. 3 herein for ACA1, or for Ip90, a subsequence thereof which comprises at least 15 nucleotides and which hybridizes to said nucleotide sequence under high stringency hybridization conditions comprising hybridization at 67°C in 2xSSC and final washing at 67°C in 1xSSC, or under medium high stringency hybridization conditions comprising hybridization at 67°C in 3xSSC and final washing at 67°C in 1xSSC, or under low stringency conditions such as 6 x SSC and 67°C and subsequent wash at 4 x SSC and 67°C, or a homologue of said nucleotide sequence which encodes a polypeptide identical to or substantially identical to a polypeptide encoded by said nucleotide sequence and which hybridizes to said nucleotide sequence under high or medium high or low stringency hybridization conditions as defined above.

An example of a hybridization procedure is described in connection with the examples.

25 In connection with the DNA fragment aspect of the present invention, the term "subsequence" is used to denote a nucleotide sequence which is derived from the nucleotide sequence shown by modifications e.g. of the types described below, and which has retained a characteristic nucleotide sequence thereof, the characteristic nucleotide sequence being a sequence which is capable of hybridizing to the nucleotide sequence in question under the conditions as explained herein. Typically, the subsequence is a part of the DNA fragment, that is, the subsequence is a number of nucleotides shorter than the DNA fragment.

In accordance with what is explained above, the DNA fragment of the invention may be one which has been modified by substitution, addition, insertion, deletion or rearrangement of one or more nucleotides in the sequence, e.g. for the purpose of establishing a sequence which, when expressed in a suitable host organism, results in the production of a polypeptide which has a substantial similarity to the OspA or OspB protein or a fragment thereof, and which has retained the immunological activity of the protein or the fragment.

35 The complete fragment shown in Fig. 3 comprises fragments coding for OspA protein and for OspB protein. One particular aspect of the invention is a DNA fragment as defined above comprising a nucleotide sequence starting at nucleotide 37 and terminating at nucleotide 851 of the nucleotide sequence shown in Fig. 3 herein encoding a cell membrane protein from B. burgdorferi, strain ACA1, or from B. burgdorferi, strain Ip90, said protein being designated OspA, or a subsequence or homologue of said nucleotide sequence, the nucleotide numbering being based on the B31 numbering in the manner shown in Fig. 3. Another particular aspect of the invention is a DNA fragment comprising a nucleotide sequence starting at nucleotide 867 and terminating at nucleotide 1877 of the nucleotide sequence shown in Fig. 3 herein encoding a cell membrane protein from B. burgdorferi, strain ACA1, or from B. burgdorferi, strain Ip90, said protein being designated OspB, or a subsequence or homologue of said nucleotide sequence, the nucleotide numbering being based on the B31 numbering in the manner shown in Fig. 3.

40 The modifications or homologues of the DNA fragments encoding OspA, OspB or a part thereof falling within the definitions herein above may be prepared, e.g. by subjecting the native DNA fragments to mutagenization, e.g. by treatment with ultraviolet radiation, ionizing radiation or a chemical mutagen such as mitomycin C, 5-bromouracil, methylmethane sulphonate, nitrogen mustard or a nitrofur, in particular so as to alter some of the properties of the gene product expressed from the mutagenized sequence substantially without changing the immunologic activity of the gene product. Especially, site-directed mutagenesis or directed mutagenesis is useful. All of these techniques are described in textbooks in the field, e.g. in (52) and (53).

The DNA sequences shown in Fig. 3 are discussed in detail in Example 1 herein.

55 Especially interesting DNA fragments are fragments which encode immunologically active parts of OspA or Osp B, i.e. the antigenic determinants or epitopes of OspA, that is, in particular, polypeptides which are capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi.

In the OspA and OspB polypeptides encoded by the DNA fragments according to the invention, polypeptide

fragments comprising sequences selected from the following:

LVSKEKNKDGKYDL,  
LVSKEKDKDGKYSL,

5

10

15

20

25

30

35

40

45

50

55



5

KGTSKNNNGSGV,  
KGTSKNTNGSGV,  
KGTSKNNNGSGT,

10

LEGVKADKSKVKL,  
LEGTKDDKSKAKL,  
LEGEKTDKSKAKL,

15

KKVTSKDKSSTEEK,  
RKVSSKDKTSTDEM,  
KKVTILKDKSSTEEK,

20

KKTKDLVFTKEN,  
KKTTQLVFTKQD,  
RKTKNLVFTKED,

25

QYDSNGTKLEGS,  
KYDSAGTNLEGT,  
KYDSAGTNLEGK,

30

AVEITKLDEIKNALK,  
AVEIKTLDELKNALK,  
AVEITTLKELKDALK,

35

DLNLEDSSKKSHQNAK,  
DQEIINSNDNTPKDSKK,  
DQDVEDLKKDQKDDSK,

40

KIFVSKEKNSSGK,  
KIFVSKEKNSAGK,  
EIFISKEKNEDDK,

45

50

KPDKSKVKLTVSAD,  
KADKTKVAMTIADD,  
KADKSKVTMLVSDD,

55

KKTGKWEDSTSTL,  
KKTATWNETTNTL,

KKTAVWNDTSSTL,

5

KNLSELKNALK,

KDLAALKAALK,

KDLEALKAALK

10 have been identified by computer analysis to be potential epitopes. Thus, such fragments and and polypeptide fragments comprising sequences which are at least 70%, preferably at least 80% and more preferably at least 90% homologous with any of the above sequences and capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi, said polypeptide fragment or fragments being smaller in size than naturally occurring OspA or OspB, are considered especially important antigenic/immunogenic polypeptide fragments which will be valuable to elicit immune responses to B. burgdorferi. Accordingly, DNA fragments encoding these polypeptide fragments are especially interesting DNA fragments according to the invention.

15 The DNA fragments according to the invention, including the DNA fragments illustrated in Fig. 3 or a sub-sequences of said fragment may be derived by screening B. burgdorferi for nucleotide sequences hybridizing to a DNA probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 3. Further, the DNA fragment sequence may be a synthetic sequence, i.e. a sequence which is prepared according to standard procedures, e.g. as described in Matthes et al., 1984 (29).

20 The DNA fragment of the invention may be used for the production of OspA, OspB or parts thereof, especially immunologically active parts thereof. For this purpose, conventional recombinant DNA techniques may be employed. Thus, techniques comprising inserting the DNA fragment of the invention or one or more parts thereof into a suitable expression vector, transforming a host organism with the vector, cultivating the organism under conditions allowing expression of the inserted sequence and harvesting the resulting gene product, 25 OspA or a part thereof, will be useful. Any of these procedures may be carried out by standard methods such as those disclosed in Maniatis et al., 1982 (30).

30 Suitable expression vectors for the production of OspA, OspB or a part thereof are vectors which is capable of replicating in a host organism when transformed therein. The vector may either be one which is capable of autonomous replication, such as a plasmid, or one which is replicated with the host chromosome, such as a bacteriophage. Examples of suitable vectors which have been widely employed are pBR322 and related vectors as well as pUC vectors and the like. Examples of suitable bacteriophages include M13 and lambda.

35 The organism harbouring the vector carrying the DNA fragment shown in Fig. 3 or part thereof may be any organism which is capable of expressing said DNA fragment. The organism is preferably a microorganism such as a bacterium. Gram-positive as well as gram-negative bacteria may be employed. Especially a gram-negative bacterium such as E. coli is useful, but also gram-positive bacteria such as B. subtilis and other types of microorganisms such as yeasts or fungi or other organisms conventionally used to produce recombinant DNA products may be used.

40 Another type of organism which may be used to express OspA, OspB or a part thereof is a higher eukaryotic organism or cell, including a plant and mammal cell. However, also higher organisms such as animals, e.g. sheep, cattle, goats, pigs, horses and domestic animals, including cats and dogs, are contemplated to be useful as host organisms for the production of OspA or a part thereof. When a higher organism, e.g. an animal, is employed for the production of OspA or a part thereof, conventional transgenic techniques may be employed. 45 These techniques comprise inserting the DNA fragment shown in Fig. 5 or one or more parts thereof into the genome of the animal in such a position that OspA or part thereof is expressed together with a polypeptide which is inherently expressed by the animal, preferably a polypeptide which is easily recovered from the animal, e.g. a polypeptide which is secreted by the animal, such as a milk protein or the like. Alternatively, the DNA fragment of the invention could be inserted into the genome of the animal in a position allowing the gene product of the expressed DNA sequence to be retained in the animal body so that a substantial steady immunization of the animal takes place.

50 When a microorganism is used for expressing the DNA fragment of the invention, the cultivation conditions will typically depend on the type of microorganism employed, and the skilled art worker will know which cultivation method to choose and how to optimize this method.

55 The production of OspA or OspB or a part thereof by recombinant techniques has a number of advantages: it is possible to produce OspA or OspB or part thereof by culturing non-pathogenic organisms or other organisms which do not affect the immunological properties of OspA or OspB or part thereof, and it is possible to produce parts of OspA or OspB which may not be isolated from B. burgdorferi strains. High quantities of OspA

or OspB or parts thereof may for instance be obtained by using high copy number vectors for cloning the DNA fragment of the invention or by using a strong promoter to induce a higher level of expression than the expression level obtained with the promoters P1 and P2 present on the DNA fragment of the invention. By use of recombinant DNA techniques for producing OspA or OspB or parts thereof, unlimited amounts of a substantially pure protein or polypeptide which is not "contaminated" with other components which are normally present in *B. burgdorferi* isolates may be obtained. Thus, it is possible to obtain a substantially pure OspA or OspB protein, i.e. OspA or OspB which is not admixed with other *B. burgdorferi* proteins which have an adverse effect when present in a vaccine or a diagnostic agent in which the OspA or OspB is an intended constituent. A substantially pure OspA or OspB protein or a polypeptide part thereof has the additional advantage that the exact concentration thereof in a given vaccine preparation is known so that an exact dosage may be administered to the individual to be immunized.

Another aspect of the invention are the non-naturally occurring polypeptides encoded by the DNA-sequences in Fig. 3 for ACA1 and Ip90. Such polypeptides will appear substantially free of peptides with which their natural counterpart would co-occur.

Thus, one embodiment of this aspect of the invention relates to a substantially pure polypeptide comprising an amino acid sequence as shown for ACA1 or Ip90 in Fig. 5, an antigenic sub-sequence of said amino acid sequence comprising at least one epitope capable of interacting with immunocompetent cells to elicit an immune response against *B. burgdorferi*, or a polypeptide having a homology of at least about 70% with said amino acid sequence, preferably a homology of at least about 80%, and in particular a homology of at least about 90%. Examples of such substantially pure polypeptides are the amino acid sequences OspA and OspB as shown for ACA1 and Ip90, respectively, and antigenic sub-sequences thereof. The subsequences will preferably be between 5 and 100 amino acids in length. The subsequences and homologues are typically prepared by recombinant techniques as described above, modifications compared to the sequences illustrated in Fig. 5 suitably being introduced by any of the DNA-modifying techniques described above, such as mutagenesis. Short synthetic polypeptide sequences according to the invention can also be prepared using DNA made by oligonucleotide synthesis, or they can be produced by solid or liquid phase peptide synthesis.

In the present context, the term "polypeptide" is used in its conventional meaning, i.e. as a sequence of amino acids. The amino acids of the sequence may optionally have been modified, after the preparation of the polypeptide, e.g. by chemical, enzymatic or another type of treatment, which does not amend or destroy the immunological activity of the polypeptide to any substantial extent. The polypeptide may be an entire protein, or a subsequence thereof. Especially interesting polypeptides are amino acid sequences comprising epitopes, i.e. antigenic properties of the polypeptide and being capable of evoking an immune response. The minimum amino acid sequence is one which at least comprises a relevant epitope of the polypeptide.

The antigenicity or immunogenicity of the polypeptides according to the invention can be assessed by usual immunisation experiments, using, e.g., rodents such as mice or rabbits as the immunized animal, or using assays, e.g. ELISA, with polyclonal or monoclonal antibodies raised in advance against *B. burgdorferi* or an immunogenic *B. burgdorferi*-related protein or polypeptide.

A particular aspect of the invention relates to synthetic polypeptide fragments at least one amino acid sequence selected from

LVSKEKNKDGKYDL,

LVSKEKDKDGKYSL,

KGTSCKNNGSGV,

KGTSCKTNGSGV,

KGTSCKNNGSGT,

LEGVKADKSKVKL,

LEGTKDDKSKAKL,

LEGEKTDKSKAKL,

5                   KKVTSKDKSSTEEK,  
                  RKVSSKDKTSTDEM,  
                  KKVTLKDKSSTEEK,

10                  KKTCDLVFTKEN,  
                  KKTQQLVFTKQD,  
                  RKTKNLVFTKED,

15                  QYDSNGTKLEGS,  
                  KYDSAGTNLEGT,  
                  KYDSAGTNLEGK,

20                  AVEITKLDEIKNALK,  
                  AVEIKTLDELKNALK,  
25                  AVEITTLKELKDALK,

                  DLNLEDSSKKSHQNAK  
30                  DQEIIINSNTPKDSKK  
                  DQDVEDLKQKDDSK

                  KIFVSKEKNSSGK,  
35                  KIFVSKEKNSAGK,  
                  EIFISKEKNEDDK,

                  KPDKSKVKLTVSAD,  
40                  KADKTKVAMTIADD,  
                  KADKSKVTMLVSDD,

45                  KKTGKWEDSTSTL,  
                  KKTATWNETTNTL,  
                  KKTAVWNDTSSTL,

50                  KNLSELKNALK,  
                  KDLAALKAALK, and  
55                  KDLEALKAALK,

and polypeptide fragments comprising sequences which are at least 70% homologous, preferably at least 80% homologous, and in particular at least 90% homologous, with any of the above sequences and capable of in-

teracting with immunocompetent cells to elicit an immune response against *B. burgdorferi*, said polypeptide fragment or fragments being smaller in size than naturally occurring OspA or OspB. As mentioned above, computer analysis has indicated that these sequences are of particular value as epitopes. The above sequences comprise sequences derived from the OspA and OspB proteins from B31 in addition to the proteins from ACA1 and Ip90, the first one in each group of two or three sequences being derived from B31, the second from ACA1, and the third from Ip90 (in the case of the first group, there is complete identity between the sequence (the second sequence of the group) derived from ACA1 and the corresponding sequence derived from Ip90).

The abbreviations of the amino acids used herein should be interpreted as follows:

Amino acid symbol	Three-letter	One letter
	abbreviation	
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

A further aspect of the present invention is an antigenic composition comprising, as an antigenic component, a polypeptide encoded by the DNA fragment as defined above or a polypeptide as defined above.

The antigenic composition may comprise a combination of polypeptides encoded from at least two of the OspA genes from B31, ACA1, and Ip90, and/or a combination of polypeptides encoded from at least two of the OspB genes from B31, ACA1, and Ip90, such as a combination of polypeptides encoded from all three of the OspA genes from the three strains mentioned, optionally combined with polypeptides encoded by one, two, or three of the OspB genes from the three strains in question. Such a composition may be useful in generating an immune response against *B. burgdorferi* irrespective of the strain of the *B. burgdorferi* spirochaete.

An example of such a composition is a composition which contains a combination of polypeptides comprising at least one polypeptide fragment selected from

5 LVSKEKNKDGKYDL,  
KGTSDKNNGSGV,  
LEGVKADKSKVKL,  
KKVTSKDKSSTEEL,  
10 KKTLDLVFTKEN,  
QYDSNGTKLEGS,  
AVEITKLDEIKNALK,  
DLNLEDSSKKSHQNAK,  
15 KIFVSKEKNSSGK,  
KPDKSKVKLTVSAD,  
KKTGKWEDSTSL,  
20 KNLSELKNALK

at least one polypeptide fragment selected from

25 LVSKEKDKDGKYSL,  
KGTSDKTNGSGV,  
LEGTKDDKSKAKL,  
RKVSSKDKTSTDEM,  
30 KKTTLQLVFTKQD,  
KYDSAGTNLEGT,  
35 AVEIKTLDELKNALK,  
DQEIINSNDNTPKDSKK,  
KIFVSKEKNSAGK,  
KADKTKVAMTIADD,  
40 KKTATWNETTNTL,  
KDLAALKAALK,

45 and at least one polypeptide fragment selected from

50

55

5 KGTSDKNNNGSGT,  
 LEGETDKSKAKL,  
 KKVTLKDKSSTEEL,  
 RKTKNLVFTKED,  
 KYDSAGTNLEGK,  
 10 AVEITTLKELKDALK,  
 DQDVEDLKDKQKDDSK,  
 EIFISKEKNEDDK,  
 KADKSKVTMLVSDS,  
 15 KKTAVWNDTSSTL,  
 KDLEALKAALK,

20 or polypeptide fragments comprising sequences which are at least 70% homologous, preferably at least 80% homologous, and in particular at least 90% homologous, with any of the above sequences, the combination being capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi of any of the strains B31, ACA1, and Ip90, said polypeptide fragment or fragments being smaller in size than naturally occurring OspA or OspB said polypeptide fragments being smaller in size than naturally occurring OspA or OspB, the size here and in the above-discussed contexts typically corresponding to only the amino acid sequences of the sizes as shown or of the same order of sizes.

25 While the strategy of the above-mentioned composition is to provide an epitope characteristic of each of the strains of B. burgdorferi, another important strategy made possible by the present invention is an antigenic composition comprising a polypeptide fragment selected from

30 LVSKEKNKDGKYDL,  
 LVSKEKDKDGKYSL,  
 35 KGTSDKNNNGSGV,  
 KGTSDKTNGSGV, and  
 KGTSDKNNNGSGT,

40 or polypeptide fragments comprising sequences which are at least 70% homologous, preferably at least 80% homologous, and in particular at least 90% homologous, with any of the above sequences and capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi of any of the strains B31, ACA1, and Ip90, said polypeptide fragment or fragments being smaller in size than naturally occurring OspA or OspB, such as discussed above.

45 As will appear from Fig. 5, there is a very high degree of identity/homology between the sequences shown immediately above in the three strains, and also other regions can be easily identified where there is a high degree of homology, those regions where there is a high degree of homology, e.g. a homology of least 70%, preferably of at least 80%, and in particular at least 90%, and where, at the same time, the homologous polypeptides are immunogenic, such as can be assessed as described above, being candidates for providing single
 50 polypeptides which are capable of eliciting a strain-independent immune response against B. burgdorferi.

A further aspect of the invention is a vaccine for immunizing mammals, including humans, against Lyme disease, the vaccine comprising an antigenic composition as discussed above and an immunogenically acceptable carrier or vehicle.

55 The carrier or vehicle may be selected from macromolecular carriers such as a polymer, e.g. a polysaccharide or a polypeptide. In addition to the carrier or vehicle, the vaccine may contain an adjuvant, such as Freund's complete or incomplete adjuvant, aluminum hydroxide, a saponin, a muramyl dipeptide, an iscome and an oil, such as a vegetable oil, e.g. peanut oil, or a mineral oil, e.g. silicone oil.

In the vaccine, the immunogenic component(s) is/are coupled to a carrier, in particular a macromolecular

carrier. The carrier is usually a polymer to which the immunogenic component(s) is/are bound by hydrophobic non-covalent interaction, such as a plastic, e.g. polystyrene, or a polymer to which the immunogenic component(s) is/are covalently bound, such as a polysaccharide, or a polypeptide, e.g. bovine serum albumin, ovalbumin or keyhole limpet hemocyanin. The carrier should preferably be non-toxic and non-allergenic. The immunogenic component(s) may be multivalently coupled to the macromolecular carrier as this provides an increased immunogenicity of the vaccine preparation. It is also contemplated that the immunogenic component(s) may be presented in multivalent form by polymerizing the immunogenic component(s) with itself.

In this regard, it may prove advantageous to couple the immunogenic component to the carrier together with one or more immunologically active molecules obtained from organisms other than B. burgdorferi so as to obtain a vaccine comprising a variety of different immunogenic determinants, being a cocktail vaccine, which may be employed for the immunization against diseases caused by other organisms, e.g. organisms responsible for relapsing fever or syphilis.

In another embodiment, a mixture of two or more single vaccines may be employed.

It is known that antibodies raised against B. burgdorferi or parts thereof evoking an immune response have a rather short lifetime in sera of animals and humans. Thus, a suitable strategy for immunizing animals and humans against Lyme disease is to periodically administer the vaccine described above to individuals subjected to contact with ticks bearing B. burgdorferi. It is contemplated that vaccination once a year such as in the springtime will provide a suitable protection of individuals in risk of B. burgdorferi infection. A suitable dose of immunogenic components for such a vaccination is 5-500 µg. However, also more irregular immunizations may be advantageous, and any immunization route which may be contemplated or shown to produce an appropriate immune response can be employed in accordance with the principle of the present invention. Suitable administration forms of the vaccine of the invention are oral administration forms, e.g. tablets, granules or capsules, subcutaneous, intracutaneous or intramuscular administration forms or forms suitable for nasal or rectal administration.

While the vaccines and compositions discussed above will elicit a humoral immune response, it is possible additionally to evoke a cellular response by incorporating, together with the polypeptide, the carrier, and the optional adjuvant, a T-cell epitope peptide. If desired, such a T-cell epitope peptide can be bonded to the above-discussed polypeptide by peptide bonds and can be encoded from the same DNA fragment which encodes the above-discussed polypeptide, the DNA fragment having been supplemented with a nucleotide sequence coding for the T-cell epitope peptide.

One interesting method for selecting or developing epitopes eliciting immune responses to B. burgdorferi is to utilize a selection pressure invoked by incubation with monoclonal antibodies and investigating sequences of the OspA and OspB proteins of the surviving variants of B. burgdorferi. This method is illustrated in Example 6.

As stated above, recombinant DNA technologies are useful for the preparation of diagnostic reagents and vaccines. Routine methods for vaccine production involve risks of obtaining unwanted side effects, e.g. due to the vaccine containing unwanted (or even unidentified) contaminants. An alternative approach to the production of new vaccines involves the insertion of one or more DNA sequences constituting one or more parts of the DNA sequence shown in Fig. 5 or parts thereof into a virus genome, e.g. into a retrovirus, vaccinia virus or Epstein-Barr virus genome, to produce a polyvalent vaccine. An especially interesting virus for the present purpose is vaccinia. Also, synthetic polypeptides which have been prepared by conventional methods, e.g. by solid or liquid phase peptide synthesis, are suitable for vaccines.

In a further aspect, the present invention relates to a non-pathogenic microorganism which carries and is capable of expressing an inserted nucleotide sequence which is the nucleotide sequence shown in Fig. 5 or part thereof for use as a live vaccine for the immunization of an animal against Lyme disease. For instance, the use of a live vaccine might be advantageous since it is presumed that vaccines based on living organisms show an excellent immunogenicity, and it is also contemplated that the use of a live vaccine will confer a life-long immunity against Lyme disease so that repeated vaccination will not be needed.

In a particularly advantageous embodiment of the live vaccine of the invention, the DNA fragment of the invention is expressed on the outer surface of the host microorganism. This provides a favourable presentation of the immunologically active part(s) of OspA recognized by the immune defense mechanisms of the animal to which the live vaccine is administered, thus provoking an appropriate immune response. One way of providing the expression of OspA or immunologically active part(s) thereof (the epitopes) on the cell surface is to fuse the DNA fragment of the invention to another nucleotide sequence encoding a surface protein or a subsequence thereof (e.g. a signal peptide) which cause the B. burgdorferi epitopes to be expressed on the outer surface of the host cell, optionally as a fused polypeptide. Examples of useful surface proteins are adhesins, fimbrial proteins, or other extracellular proteins.

The microorganism used for live vaccines should be a non-pathogenic microorganism, e.g. a non-patho-



genic *E. coli*, which may be able to establish itself in the animal body. A microorganism which may prove especially useful as a live vaccine may be the *B. burgdorferi* in itself, which as explained above inherently expresses OspA on the surface of the cell. The use of *B. burgdorferi* for a live vaccine requires, however, that the *B. burgdorferi*, has been altered so as to not cause any illness when used as a live vaccine. This alteration or modification may be carried out in any suitable manner, e.g. by mutagenization, chemical, enzymatic or heat treatment, or by another equivalent treatment resulting in an attenuated *B. burgdorferi* cell.

#### Hybridization of DNA

DNA, e.g. present on nitrocellulose filters, are wetted in 2 x SSC [1 x SSC: 0.15 M NaCl, 0.0015 M Na<sub>3</sub>-citrate, pH 7.0] and placed in a heat-sealed plastic bag with pre-warmed 67°C) prehybridization solution. Prehybridization takes place for 2 h at 67°C, the bag being gently shaken. The solution is exchanged with pre-warmed (67°C) hybridization solution, the radioactive probe is added and hybridization is carried out at 67°C for 18 h. The bag is gently shaken to ensure constant movement of the liquid over the nitrocellulose filters. After hybridization, a washing procedure is carried out.

The radioactive probe is prepared by use of known methods, e.g. as described by Sambrook et al., on the basis of the DNA sequence shown in Sequence Listing 1 or a part thereof, especially a coding part such as the nucleotides corresponding to amino acids 1-210 or an effective subsequence of the DNA sequence as defined above.

The prehybridization and hybridization solutions used are: 10 x Denhardt's, 4 x SSC, 0.1% SDS, 10 µg/ml polyA, 50 µg/ml of denatured DNA to be analysed and the denatured (heat) radioactive probe. The filters are washed in pre-warmed (67°C) solutions: 10 x Denhardt, 2 x SSC, 0.1% SDS for 2 x 15 min. and 1 x SSC, 0.1% SDS for 4 x 15 min. The filters are air-dried and covered with Vita-Wrap, and X-ray film is exposed to the filters for 3 h to 3 weeks with and without intensifying screens.

#### EXAMPLE 1

##### Isolation and sequence analysis of the OspA and OspB genes

#### Bacterial strains

The *Borrelia burgdorferi* strains used were the American reference strain B31 (ATCC 35210), the Swedish isolate ACAI isolated from a patient with acrodermatitis chronica migrans (1), and the Ip90 strain which is isolated from *I. persulcatus* from the Soviet union and was kindly provided by E. I. Korenberg and V. N. Kryuchevnikov of the Gamaleya Institute, Moscow (27).

#### Media and culturing conditions

The *B. burgdorferi* strains were cultivated in BSK II medium as previously described (2). The *Escherichia coli* strains DH5α (BRL, Gaithersburg, Md. USA) and Y1090 (24) were used for propagation of recombinant plasmids and for growth of λgt11 phage gene library, respectively.

#### Construction and screening of λ-gt11 and pUC18 plasmid *B. burgdorferi* gene libraries

*B. burgdorferi* strains were cultured in 400 ml modified BSKII-medium (2) at 34°C and harvested at late mid-log phase. The DNA was extracted and purified as previously described (23). The ACAI DNA was partially digested by Sau3AI and fragments 4-8 kb in size were isolated from a 0.7% agarose gel. The fragments were then ligated into BamHI-cut λ-gt11 arms and packaged into phage heads as described by the vendor (SDS-Promega, Falkenberg, Sweden). The λ-phages were propagated in *E. coli* Y 1090 and screening, by DNA hybridization, of the λ-library was according to standard methods (24). The oligonucleotides used for screening, J1, J2, and J3, were synthesized from the previously published *B. burgdorferi* B31 *osp*-operon nucleotide sequence (14). All nucleotides used for screening of phage and plasmid libraries and primers for nucleotide sequencing are shown in Table 1. The oligonucleotides were end labelled with <sup>32</sup>P-γ-dATP, as previously described (31), and purified on a Sephadex G-50 (Pharmacia, Uppsala, Sweden) spin column. The hybridization was performed at 37°C, i.e. medium stringency, as some differences in the nucleotide sequence between the different strains could be expected. The phage DNA was extracted as previously described (28). Purified λ-DNA was further subcloned into the EcoRI site of pUC18 according to standard methods (30).

A plasmid gene library of *B. burgdorferi* Ip90 DNA was constructed by partial Hind III digestion of total DNA,

in which 1 u enzyme was incubated with 100 ng DNA at 37°C for 15 min. The reaction was terminated by a phenol:chloroform (1:1) extraction. After ethanol precipitation, the fragments were ligated with 30 ng HindIII digested pUC18 plasmid DNA at 16°C for 4 h. The plasmid were transformed into competent *E. coli* DH5 $\alpha$  cells. An additional plasmid gene library was constructed by complete EcoRI digestion of IP90 DNA and cloning into the EcoRI site of the plasmid pUC18.

The *B. burgdorferi* Ip90 plasmid gene library was screened with a 259 bp *osp*-fragment. This *ospA* fragment was obtained by PCR amplification of a DNA segment located at the end of the *ospA* gene, using the J1 and K1 oligonucleotides as primers (Table 1). This fragment corresponds to a fragment between nucleotide positions 529 to 788 of the *B. burgdorferi* B31 *ospA* gene. The conditions for the PCR amplification were as follows; 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 67 mM Tris-HCl (pH 8.8 at 25°C), 6.7 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 200  $\mu$ M each of dATP, dGTP, dCTP, and dTTP, 170  $\mu$ g per ml bovine serum albumin, 5 pmol of each primer, about 10 pg total Ip90 DNA and 1 u of BioLabs Taq polymerase. Reactions were performed in a volume of 50  $\mu$ l, overlaid with 40  $\mu$ l of mineral oil. During the first 5 cycles, denaturation was done at 94°C for 2 min, annealing at 45°C for 1 min and elongation at 72°C for 1 min. In the next 30 cycles, the denaturation time was shortened to 39 sec and annealing temperature raised to 55°C, otherwise the conditions were the same. The PCR fragments was labelled with <sup>32</sup>P- $\alpha$ -dATP using an oligo-labelling kit (Amersham, Buckinghamshire, UK) and used to screen the HindIII library for *osp*-gene containing clones. The screening was performed as described (30), and the hybridization was performed at high stringency (60°C).

#### Nucleotide sequence analysis

Deletion libraries in one direction were constructed for the full length clone of ACAI and the shorter or the Ip90 clones as described earlier (21). Mini plasmid preparations were either performed according to the boiling method (30) or by a CsCl method previously described (39). Nucleotide sequencing was performed using the dideoxy chain-termination method described by Sanger et al. with the use of Pharmacia<sup>TM</sup> Sequencing<sup>TM</sup> kit (Pharmacia, Uppsala, Sweden). The complete sequence for both strands was determined. From the nucleotide sequences obtained, internal primers were synthesized to enable sequencing of the other strand plus the beginning of the Ip90 *osp*-operon. The sequences obtained from the DNA deletion sequencing method were assembled using the GENEUS (20) software for VAX computers (Digital Equipment Corporation). Additional nucleotide sequence analyses were performed using the University of Wisconsin GCG Sequence Analysis software for the VAX computer, and PC-Gene (Genofit) for the personal computers.

#### In vitro transcription analysis

Transcriptional analysis was performed on total mRNA isolated from *B. burgdorferi* B31 using a method previously described (31). The isolated and purified mRNA was subjected to primer extension in an *in vitro* reaction using Avian myeloblastosis virus reverse transcriptase (Lif Sciences, Fla. USA) as described previously with minor modifications (13, 17, 48). The primer extension reaction with RNA was carried out with [ $\alpha$ -<sup>32</sup>P]-dATP. The primer used in the primer extension reaction was the reverse complement of nucleotides 128 to 160 in Figure (3). Only full-length mRNA polymer was synthesized, the size of the transcript was compared with a regular Sanger dideoxy DNA sequence on plasmid pTHR44 obtained when using the same oligonucleotide primer. In the DNA sequencing reactions [ $\alpha$ -<sup>35</sup>S]-dATP was used.

#### EXAMPLE 2

##### Cloning and nucleotide sequence analysis of *osp*-operons from *B. burgdorferi* isolates ACAI and Ip90

##### Sequencing analysis

The *osp*-operons from the Swedish *B. burgdorferi* strain ACAI was isolated from a  $\lambda$ gt11 library. The isolation of the ACAI *osp*-operon was performed by screening with a mixture of three oligonucleotides, J1, J2 and J3, which were synthesized from the nucleotide sequence of the previously published *B. burgdorferi* B31 *osp*-operon. One positive  $\lambda$ -clone containing the ACAI *ospA* and *ospB* genes was isolated and characterized by restriction endonuclease mapping. The isolated *osp* operon was subcloned into pUC18 and the genetic organization of the genes was confirmed with the same three oligonucleotides, J1, J2, and J3. The *osp*-operon of strain Ip90 was cloned for a pUC18 plasmid library using Hind III, partially digested, total Ip90 *B. burgdorferi* DNA. One positive clone containing almost the entire Ip90 *osp*-operon except the first 175 bp of *ospA* was obtained. An oligonucleotide, J4, was constructed from the start of this Hind III clone. This oligonucleotide,

J4, was used to pick up a clone containing the whole *Ip90* *osp*-operon from a pUC18 plasmid library of completely *EcoRI* digested *Ip90* DNA.

The *osp*-operons were sequenced and their nucleotide sequences of the *osp*-operons of strains ACAI and *Ip90* are shown in Figure 3. The obtained nucleotide sequences are, in the Figure, aligned and compared to the previously published nucleotide sequences of the *ospA* and *ospB* genes of strain B31 (14) and the *ospA* sequences from the strains Z57 (45) and N40 (19). The percentage DNA identity of the respective *ospA* and *ospB* genes are shown in Table 2 and 3. The results of the nucleotide sequence comparison revealed that the *ospA* genes from strains ACAI and *Ip90* exhibited a sequence identity of 85% with the *ospA* genes of strain B31. When compared to each other, the *ospA* genes of ACAI and *Ip90* also showed an 85% similarity. In contrast, the two previously published *ospA* sequences from strains Z57 and N40 were almost identical to the *ospA* sequence of strain B31 (>99%). The *ospB* sequences of strain ACAI and *Ip90* were 79% identical to the *ospB* gene of strain B31 and 81% identical compared to each other. Further analysis of the *osp*-genes of strain ACAI and *Ip90* revealed that *ospA* and *ospB* genes are organized in one operon, separated by just a few basepairs. The *osp*-operons in these two strains are also preceded by a control region consisting of a  $\sigma$ -70 promotor and a Shine and Dalgarno ribosomal binding site, as depicted in figure 3 (37). In order to determine the exact transcriptional start point of the *osp*-operon, an *in vitro* primer extension was performed on isolated total messenger RNA from *B. burgdorferi* B31 (Figure 4). The *in vitro* transcription analysis identified the transcriptional start site as the G at position +1 (Figure 3). This transcriptional start site is situated 36 bp upstream of the AUG translational start codon.

### EXAMPLE 3

#### Sequence analysis of the translated OspA and OspB proteins of strains ACAI and *Ip90*.

The translated products of the *ospA* genes of ACA1 and *Ip90* were compared with the deduced translation products of strains B31, ZS7, and N40. The comparison of the different OspA proteins in an optimal alignment are shown in Figure 5A. The deduced OspA protein for ACA1 is 273 amino acids long with a theoretical molecular weight of 29,629 and for *Ip90* it is 274 amino acids long predicting a protein with a molecular weight 29,673. The three different *ospB* gene products are compared in Figure 5B. The deduced OspB proteins have a molecular weight of 32,432 coded by 299 amino acids for ACA1, and 32,105 coded by 294 amino acids for *Ip90*. From the amino acid sequence comparison of the OspA and OspB proteins it is evident that the start of the OspA is very conserved between the different strains, while the middle and the C-terminal parts of the proteins show a higher degree of variation. In the OspB protein the same overall variability in the sequence is seen. From the deduced amino acid sequence of the OspA and OspB proteins of *B. burgdorferi* strain B31 the sequence similarity with a prokaryotic lipoproteins was shown (14). The deduced OspA and OspB proteins of *B. burgdorferi* strains ACA1 and *Ip90* also contain the typical consensus tetrapeptide (LXYC) in their peptides. The possible signal peptidase II recognition sites of the OspA and OspB proteins are indicated in the Figures (5A and 5B).

### EXAMPLE 4

#### Extraction of *B. burgdorferi* proteins, SDS-PAGE, and Western blotting.

The cells were grown in 200 ml at 34°C and harvested in mid-log phase (approximately  $2$  to  $4 \times 10^8$  cells per ml) by centrifugation at 8,000 g for 20 min. The cells were washed twice in PBS-5 mM  $MgCl_2$ , and the pellets were resuspended in 2 ml PBS. To prepare soluble proteins, the cells were sonicated 4 times 30 sec in an ice bath by using a Branson Sonifier cell disrupter B15 at setting 3. After centrifugation at 10,000 g for 30 min, the supernatant was collected and the amount of protein was determined by using the Bio-Rad protein assay (Bio-Rad, Munich, Germany).

SDS-PAGE was performed essentially as described before (10) using either 12.5% or 15% acrylamide running gels and 4% acrylamide stacking gels. In each lane 10 to 15  $\mu$ g protein was added. The gels were either fixed and stained by Coomassie brilliant blue (Sigma chemical, Saint Louis, Mo, USA) or processed for immunoblotting.

Molecular weight standards were obtained from Pharmacia (Uppsala, Sweden) and included proteins ranging from 14.4 to 94 kDa. The proteins were transferred to immobilon filters (Millipore Corporation, Bedford, Ca, USA) by electroblotting at 0.8 mA over  $cm^2$  for 45 min. The non-specific binding on the filters was blocked by incubation with 5% milk powder in PBS over night. The filters were then incubated with antibodies (1:20 or 1:25 dilution in 2.5% milk powder in PBS), washed 3 times 5 min in PBS-0.05% Tween 20 and then incubated

with an appropriate peroxidase labeled monoclonal antibody, Mab (1:500 dilution in 2.5% milk/PBS) for 1 hour. Bound antibodies were then visualized by adding 5-bromo-4-chloro-3-indolyphosphate as peroxidase substrate. All incubations were performed during continuous shaking. The Mab's used were the anti-OspA antibodies H5332 (10) and the H3TS (7) and the anti-OspB antibody H6831 (9). A polyclonal antiserum raised against whole cell lysates from the Swedish *B. burgdorferi* tick isolate G152 (44), a gift from Mats Karlsson, Danderyd hospital, Stockholm, Sweden, was used in Western blotting to show the presence of *B. burgdorferi* major outer surface proteins.

#### EXAMPLE 5

##### Biochemical and immunochemical characterization of the OspA and OspB proteins of *B. burgdorferi* strains ACA1 and Ip90.

Whole cell protein extracts prepared from *B. burgdorferi* strains B31, ACA1 and Ip90 separated on a 12.5% SDS-PAGE are shown in Figure 1. These three different *B. burgdorferi* isolates were obtained from different geographical locations. The three strains show different apparent molecular weights for both the major outer surface proteins OspA and OspB. This result was also found earlier, when comparing the strains B31 and ACA1 (8). The molecular weights of the respective OspA and OspB proteins as determined from the SDS-PAGE are as follows: for B31; 31 kD and 34 kD, for ACA1; 32 kD and 36 kD, and for Ip90; 33.5 kD and 34 kD. The Osp proteins from the three different isolates were further characterized by western blot analysis using different monoclonal antibodies directed against the OspA and OspB proteins of *B. burgdorferi* B31. In a western blot using the OspA specific MAb H5332 (Fig. 2A), a protein extract from strain B31 strongly bound to this antibody, whereas the ACA1 OspA protein only reacted weakly. No protein from strain Ip90 reacted with the MAb H5332. When the OspA specific MAb H3TS (Fig. 2B) or OspB specific MAb H6831 (Fig. 2C) was used only the OspA and OspB proteins of strain B31 reacted, the Osp proteins of ACA1 and Ip90 did not react with these two monoclonal antibodies indicating a variability of the OspA and OspB proteins in these three strains. When using a polyvalent polyclonal antiserum raised against whole cell *B. burgdorferi* (Figure 2D), all three strains reacted with 10 to 15 different proteins. In all strains the strongest hybridization signal was against a protein of the size of the OspA protein. The apparent molecular weights of these presumed OspA proteins are, as calculated from the western blot, 31 kD (B31), 32 kD (ACA1), and 33.5 kD (Ip90), respectively.

#### EXAMPLE 6

##### Identification of epitopes

##### Materials

*B. burgdorferi* organism of the strains B31, ACA1 and Ip90

Monoclonal antibodies directed to OspA and OspB, H5332 and H3TS, respectively.

##### Methods

Incubating *B. burgdorferi* in the presence of one of the monoclonal antibodies at 34°C in BSKII broth culture medium, the growth of the spirochates is inhibited. After 1-7 days variants which are not inhibited by the antibody begin to grow. These antibody-resistant organisms have changes in the OspA or OspB protein. They are mutants selected with monoclonal antibodies.

The epitopes against which the growth-inhibiting antibodies are directed will be identified by comparing the deduced amino acid sequence of the original strain with the deduced amino acid sequence of the mutant.

The deduced amino acid of the mutant OspA proteins will be determined by DNA sequencing of amplified DNA from the polymerase chain reaction.

Table 1. Oligonucleotides used for screening of phage and plasmid gene libraries, nucleotide sequencing of the *osp*- operons, and primer extension analysis

Oligomer	Origin	Position*	Sequence 5' to 3'
J1	B31	788-760	TTTGAGTCGTATTGTTGTACTGTAATTGT
K1	B31	529-556	TATGTTCTTGAAGGAACTCTAACTGCTG
J2	B31	337-312	GTGTGGTTTGACCTAGATCGT
J3	B31	1237-1217	AGGTTACTGTGTTTAAATCAG
P1	pUC19	334-317	ACGCCAGGGTTTCCAG
P2	pUC19	172-189	GTGTGGAATTGTGAGCGG
I1	Ip90	278-256	GTTTTTCACCTTCAAGTGTTC
B1	B31	-67-(-48)	TTATTATCATTTTATTTTTT
B2	B31	75-54	GGCTAATATTAGACCTATTCCC
B3	B31	1084-1066	ATTTTTTCTTTGCTTAC
B4	B31	1066-1084	GTAAGCAAAGAAAAAAT
B5	B31	1425-1407	TAGAGTTTCTACTGCTTTT
B6	B31	1407-1425	TACAAAAGCAGTAGAACTC
B7	B31	1603-1622	TTAACAATTAGTGCTGACAG
B8	B31	1642-1661	GTGTTCTTAACAGATGGTAC
B9	B31	1661-1642	GTACCATCTGTTAAGAACAC
B10	B31	160-128	CAAGAAGTTTCATTTACCAGGCAATCTACTG
A1	ACAI	140-120	GGCAAATCTACTGAAGCGCTG
A2	ACAI	202-223	GCAACAGTAGACAAGATTGAGCT
A3	ACAI	452-472	GAGAAAATGGAACCAAACCTTG
A4	ACAI	525-502	TTTTAAACTTCTTTAGCTTTTCC
A5	ACAI	669-689	AAAAACTGGCGCATGGGATTC
A6	ACAI	840-821	AAGTTCATCAAGTGTTTTAA
A7	ACAI	968-992	TATAAACTCAGACAATACACC
A8	ACAI	1176-1156	ACCTTCAAGCTTGCCAGATCC
A9	ACAI	1291-1313	CAAGGGTCAGTAATAAAGAATC
A10	ACAI	1802-1779	CCTACAAAGGTATTAGCCGA

\* All positions are derived from the *B. burgdorferi* B31 *ospA* and *ospB* sequence in Figure 3, except for the pUC19 sequences, which are derived from the EMBL data base sequence of pUC19 plasmid.

Table 2. DNA-sequence identity (percent) between the *ospA*-genes from different *Borrelia burgdorferi* isolates.

	B31	N40	ZS7	ACA1	IP90
B31	*				
N40	99,8	*			
ZS7	99,5	99,8	*		
ACA1	85	85	85	*	
IP90	86	86	85	86	*

Table 3. DNA-sequence identity (percent) between the *ospB*-genes from different *Borrelia burgdorferi* isolates.

	B31	ACA1	IP90
B31	*		
ACA1	79	*	
IP90	79	81	*

#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** Coomassie blue stained 12.5% SDS-PAGE of whole cell lysates of *B. burgdorferi*. Lane A: whole cell extract of *B. burgdorferi* strain B31, lane B: strain ACA1 and lane C: strain Ip90. Lane S: both on left and right in the Figure shows the sizes of the molecular weight standards ( $\times 10^3$ ).

**Figure 2a and b** Western blot analysis using different monoclonal or polyclonal sera reacting with different epitopes of *B. burgdorferi*. The immunoblots were made from the same type of SDS-PAGE as in Figure 1, with B31 whole cell preparation in lane A, ACA1 in lane B, and Ip90 in lane C. The different sera used are as follows; In 2A H5332, a monoclonal antibody reacting with an epitope of the OspA protein. 2B) H3TS, an OspA monoclonal antibody reacting with a different epitope than H5332. 2C) H6831, a monoclonal antibody directed against the OspB protein of *B. burgdorferi* B31. 2D) A polyvalent polyclonal antisera raised against whole cell lysates from the Swedish *B. burgdorferi* tick strain G152.

**Figure 3 a, b, c, and d** Comparison of the nucleotide sequences of the *osp*-operons from *B. burgdorferi* strains B31, ACA1 and Ip90. The OspA sequences from ZS7 and N40 are also included. The OspA and OspB sequences of *B. burgdorferi* strain B31 are shown in the top row (14). Hyphens indicate homology between the OspA and OspB genes of the different strains, capital letters indicate differences between the Osp genes, and gaps missing (or inserted) bases. The -10 and -35 regions for the promotor are indicated by bold types and the ribosomal binding sites (Shine Dalgarno sequences) are underlined.

**Figure 4** Determination of the transcriptional start of the *B. burgdorferi* B31 *osp*-operon. The figure shows the primer extension of the *osp* mRNA together with the corresponding sequence ladder of the *osp* DNA. The -10 region, the transcriptional start base (+1), ribosomal binding site (RBS) and the two first amino acid codons (Met and Lys) are indicated to the right in the figure.

**Figure 5** Comparison of the deduced amino acid sequence of the *B. burgdorferi* OspA and OspB proteins. A) Comparison of the OspA proteins of strains B31, ZS7, N40, ACA1 and Ip90. All sequences are compared and aligned to the *B. burgdorferi* B31 sequence (14). Capital letters indicate differences between the strains, hyphens indicate homologies, and gaps indicate missing amino acids. The possible signal peptidase cleavage site are indicated with bold letters in the figure. B) The deduced amino acid sequence of the OspB proteins of the *B. burgdorferi* strains ACA1 and Ip90 compared to the OspB protein of *B. burgdorferi* B31.

## REFERENCES

1. Åsbrink, E. & A. Hovmark. 1985. Successful cultivation of spirochetes from skin lesions of patients with erythema chronicum migrans Afzelius and acrodermatitis chronica atrophicans. *Acta. Path. Microbiol. Immunol. Scand. Sect. B*, 93: 161-163.
2. Barbour, A.G. 1984. Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med.* 57: 71-75.
3. Barbour, A.G. 1984. Immunochemical analysis of Lyme disease spirochetes. *Yale J. Biol. Med.* 57: 581-586.
4. Barbour, A.G. 1988. Plasmid Analysis of Borrelia burgdorferi, the Lyme Disease Agent. *Journal of Clinical Microbiology* 26: 475-478.
5. Barbour, A.G. 1989. Antigenic variation in relapsing fever Borrelia species: genetic aspects. In Berg, D.E. & Howe, M.M. (eds): *Mobile DNA*, Washington, D.C. American Society for Microbiology, pp. 783-789.
6. Barbour, A.G. & C.F. Garon. 1987. Linear plasmids of the bacterium Borrelia burgdorferi have covalently closed ends. *Science* 237: 409-411.
7. Barbour, A.G., R.A. Heiland & T.R. Howe. 1985. Heterogeneity of major proteins in Lyme disease borreliae: a mMolecular analysis of North American and European isolates. *J. Infect. Dis.* 152: 478-484.
8. Barbour, A.G. & M.E. Schrupf. 1986. Polymorphism of major surface proteins of Borrelia burgdorferi. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A*. 263: 83-91.
9. Barbour, A.G., S.L. Tessier & S.F. Hayes. 1984. Variation in a major surface protein of Lyme disease spirochetes. *Infect. Immun.* 45: 94-100.
10. Barbour, A.G., S.L. Tessier & W.J. Todd. 1983. Lyme disease spirochetes and *Ixodes* ticks share a common surface antigenic determinant defined by a monoclonal antibody. *Infect. Immun.* 41: 795-804.
11. Barbour, A.G., N. Burman, C.J. Carter, T. Kitten & S. Bergström. 1991. Variable antigen genes of the relapsing fever agent Borrelia hermsii are activated by promoter addition. *Mol. Microbiol.* 5: 489-493.
12. Barbour, A.G., C.J. Carter, N. Burman, C.S. Freitag, C.F. Garon & S. Bergström. 1991. Tandem insertion sequence-like elements define the expression site for variable antigen genes of Borrelia hermsii. *Infect. Immun.* 59: 390-397.
13. Bergström, S., K. Robbins, M. Koomey & J. Swanson. 1986. Piliation control mechanisms in Neisseria gonorrhoeae. *Proc. Natl. Acad. Sci. USA* 83: 479-486.
14. Bergström, S., V.G. Bundoc & A.G. Barbour. 1989. Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochete Borrelia burgdorferi. *Mol. Microbiol.* 3: 479-486.
15. Brandt, M.E., B.S. Riley, J.D. Radolf & M.V. Norgard. 1990. Immunogenic integral membrane proteins of Borrelia burgdorferi are lipoproteins. *Infect. Immun.* 58: 983-991.
16. Bundoc, V.G. & A.G. Barbour. 1989. Clonal polymorphism of outer membrane protein OspB of Borrelia burgdorferi. *Infect. Immun.* 57: 2733-2741.
17. Burman, N., S. Bergström, B.I. Restrepo & A.G. Barbour. 1990. The variable antigens Vmp7 and Vmp21 of the relapsing fever bacterium Borrelia hermsii are structurally analogous to the VSG proteins of the African trypanosome. *Mol. Microbiol.* 4: 1715-1726.
18. Craft, J.E., D.K. Fischer, G.T. Shimamoto & A.C. Steere. 1986. Antigens of Borrelia burgdorferi recognized during Lyme disease. Appearance of a new IgM response and expansion of the IgG response late in illness. *J. Clin. Invest.* 78: 934-939.
19. Fikrig, E., S.W. Barthold, F.S. Kantor & R.A. Flavell. 1990. Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. *Science* 250: 553-556.
20. Harr, R., P. Fällman, M. Häggström, L. Wahlström & P. Gustafsson. 1986. GENEUS, a computer system for DNA and protein sequence analysis containing an information retrieval system for the EMBL data library. *Nucl. Acids Res.* 11: 273-284.
21. Hoheisel, J. & F.M. Pohl. 1986. Simplified preparation of unidirectional deletion clones. *Nucl. Acids Res.* 14: 3605.
22. Howe, T.R., F.W. LaQuier & A.G. Barbour. 1986. Organization of genes encoding two outer membrane proteins of the Lyme disease agent within a single transcriptional unit. *Infect. Immun.* 54: 207-212.
23. Howe, T.R., L.W. Mayer & A.G. Barbour. 1985. A single recombinant plasmid expressing two major outer surface proteins of the Lyme disease spirochete. *Science* 227: 645-646.
24. Huynh, T.U., R.A. Young & R.W. Davis. 1985. Construction and screening cDNA libraries in  $\lambda$ gt10 and  $\lambda$ gt11. In *DNA Cloning*, Volume 1, ed. Glover, D.M. IRL Press Limited, Oxford, England, pp. 56-110.
25. Inouye, M. & S. Halegoua. 1980. Secretion and membrane localization of proteins in Escherichia coli. *Crit. Rev. Biochem.* 7: 339-371.
26. Jameson, B.A. & H. Wolf. 1988. The antigenic index: a novel algorithm for predicting antigenic deter-

- minants. *Comput. Appl. Biosci.* 4: 181-186.
27. Kyruchevnikov, V.N., E.I. Korenberg, S.V. Scherbakov, Yu.V. Yovalevsky & M.L. Levin. 1988. Identification of Borrelia isolated in the USSR from Ixodes persulcatus schulze ticks. *J. Microbiol. Epidemiol. Immunobiol.* 12: 41-44.
- 5 28. Loenen, W.A.M. & W.J. Brammer. 1980. A bacteriophage lambda vector for cloning large DNA fragments made with several restriction enzymes. *Gene* 20: 249-259.
29. Malloy, D.C., R.K. Nauman & H. Paxton. 1990. Detection of Borrelia burgdorferi using the polymerase chain reaction. *J. Clin. Microbiol.* 28: 1089-1093.
30. Maniatis, T.E., E.F. Fritsch & J. Sambrook. 1982. *Molecular cloning: A laboratory manual*. Cold Spring Harbor, New York, Cold Spring Harbor, N.Y. USA, Laboratory press.
- 10 31. Meier, J.T., M.I. Simon & A.G. Barbour. 1985. Antigenic variation is associated with DNA rearrangements in a relapsing fever borrelia. *Cell* 41: 403-409.
32. Nielsen, S.L., K.K.Y. Young & A.G. Barbour. 1990. Detection of Borrelia burgdorferi DNA by the polymerase chain reaction. *Mol. Cell. Probes.* 4: 73-79.
- 15 33. Plasterk, R.H.A., M.I. Simon & A.G. Barbour. 1985. Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium Borrelia hermsii. *Nature* 318: 257-263.
34. Postic, D., C. Edlinger, C. Richaud, F. Grimont, Y. Dufresne, P. Perolat, G. Baranton & P.A.D. Grimont. 1990. Two genomic species in Borrelia burgdorferi. *Res. Microbiol.* 141: 465-475.
- 20 35. Rosa, P.A. & T.G. Schwan. 1989. A specific and sensitive assay for the Lyme disease spirochete Borrelia burgdorferi using the polymerase chain reaction. *J. Infect. Dis.* 160: 1018-1029.
36. Rosa, P.A., D. Hogan & T.G. Schwan. 1991. Polymerase chain reaction analyses identify two distinct classes of Borrelia burgdorferi. *J. Clin. Microbiol.* 29: 524-532.
37. Rosenberg, M. & O. Court. 1979. Regulatory sequences involved in the promotion and termination of transcription. *Ann. Rev. Genet.* 13: 256-275.
- 25 38. Sanger, F., S. Nicklen & A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74: 5463-5467.
39. Saunders, S.E. & J.F. Burke. 1990. Rapid isolation of miniprep. DNA for double strand sequencing. *Nucl. Acids Res.* 18: 4948.
- 30 40. Schaible, U.E., M.D. Kramer, K. Eichman, M. Modolell, C. Museteanu & M.M. Simon. 1990. Monoclonal antibodies specific for the outer surface protein A (OspA) of Borrelia burgdorferi prevent Lyme borreliosis in severe combined immunodeficiency (scid) mice. *Proc. Natl. Acad. Sci. USA* 87: 3768-3772.
41. Schubach, W.H., S. Mudri, R.J. Dattwyler & B.J. Luft. 1991. Mapping antibody-binding domains of the major outer surface membrane protein (OspA) of Borrelia burgdorferi. *Infect. Immun.* 59: 1911-1915.
- 35 42. Schwan, T.G. & W. Burgdorfer. 1987. Antigenic changes of Borrelia burgdorferi as a result of in vitro cultivation. *J. Infect. Dis.* 156: 852-853.
43. Schwan, T.G., W. Burgdorfer & C.F. Garon. 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, Borrelia burgdorferi, as a result of in vitro cultivation. *Infect. Immun.* 56: 1831-1836.
44. Stiernstedt, G. 1985. Tick-borne Borrelia infection in Sweden. *Scand. J. Infect. Dis. Suppl.* 45: 1-70.
- 40 45. Wallich, R., U.E. Schaible, M.M. Simon, A. Heiberger & M.D. Kramer. 1989. Cloning and sequencing of the gene encoding the outer surface protein A (OspA) of a European Borrelia burgdorferi isolate. *Nucl. Acids Res.* 17: 8864.
46. Williams, J.G. & P.J. Mason. 1985. Hybridization in the analysis of RNA. In *Nucleic acid hybridization*. Hames, B.D. & S.J. Higgins (eds), Oxford: IRL Press, pp. 139-160.
- 45 47. Wilske, B., V. Preac-Mursic, G. Schierz, R. Kuhbeck, A.G. Barbour & M. Kramer. 1988. Antigenic variability of Borrelia burgdorferi. *Ann. N.Y. Acad. Sci.* 539: 126-143.
48. von Gabain, A., J.G. Belasco, J.L. Schottel, A.C.Y. Chang & S.N. Cohen. 1983. Decay of mRNA in Escherichia coli: investigation of the fate of specific segments of transcripts. *Proc. Natl. Acad. Sci. USA* 80: 653-657.
- 50 49. von Heijne, G. 1983. Patterns of amino acids near signal sequence cleavage sites. *Eur. J. Biochem.* 133: 17-21.
50. Wu, H.C. & M. Tokunaga. 1986. Biogenesis of lipoproteins in bacteria. In *Current Topics in Microbiology and Immunology*, Vol. 125, pp. 127-157.
51. Arnheim, N. & Levenson, C.H. 36, October 1, 1990 C&EN special report. Polymerase Chain Reaction.
- 55 52. Sambrook, J. et al. *Molecular Cloning, a laboratory manual*.
53. Old, R.W. and Primrose, S.B. *Principles of Gene Manipulation, a textbook*.
- We hereby incorporate by reference any patents or publications cited herein.



## Claims

1. A method for detecting the presence of a B. burgdorferi organism in animals, including humans, the method comprising subjecting a specimen from the animal, such as a body fluid, such as blood, serum, cerebrospinal fluid, synovial fluid, pericardial fluid or urine, or a tissue biopsy, or, when the animal is an arthropod, the whole animal, to PCR-DNA analysis which either
  - A) uses a nucleotide primer sequence which comprises at least 11 nucleotides, the primer sequence being identical or substantially identical to a sub-sequence of at least two of the three DNA sequences derived from B. burgdorferi strains B31, Ip90 and ACA1, respectively, shown in Fig. 3 herein,
  - B) uses a combination of at least two nucleotide primer sequences, each of which comprises at least 11 nucleotides, one sequence being identical or substantially identical to a sub-sequence from the OspA and OspB DNA from a first B. burgdorferi strain as shown in Fig. 3 herein, another sequence being identical or substantially identical to a sub-sequence from the OspA and OspB DNA from another of the B. burgdorferi strains as shown in Fig. 3 herein, and optionally a third sequence which is identical or substantially identical to a sub-sequence from the third of the B. burgdorferi strains as shown in Fig. 3 herein,
 the substantial identity being such that the said primer sequence will hybridize to the sub-sequence under conventional hybridization conditions (reference Sam Brooks), or under high stringency hybridization conditions comprising hybridization at 67°C in 2xSSC and final washing at 67°C in 1xSSC, or under medium high stringency hybridization conditions comprising hybridization at 67°C in 3xSSC and final washing at 67°C in 1xSSC.
2. A method according to claim 1 wherein the primer sequence comprises at least 13 nucleotides, such as at least 15 nucleotides, and preferably at least 18 nucleotides.
3. A method according to claim 1 A) or 2 wherein the subsequence in all three of the strains B31, Ip90 and ACA1 is identical or substantially identical, the substantial identity being as defined in claim 1.
4. A method according to claim 1 A), 2 or 3 wherein the primer sequence comprises a sequence which is identical or substantially identical to one of the following sequences (or one of their complementary sequences):

5' -GTATTAAGTTATATTAATAT-3'

5' -AAAAGGAGAATATATTATGA-3'

5' -AAAAATATTTATTGGGAATA-3'

5' -GGAAAAGCTAAAGAGGTTTTAAAA-3'

5' -ACTTCAACTTTAACAATTA-3'

5' -AATAAGGAGAATTTATGA-3'

5' -AAAAAACTAAA-3'

the substantial identity being as defined in claim 1.

5. A method according to any of claims 1-4, wherein a set of primers is used, the first primer of the set being a primer as defined in any of claims 1A and 2-4, the second primer comprising a sequence which is identical or substantially identical to a subsequence of a strand complementary to OspA or OspB DNA from

at least one of B. burgdorferi strains B31, Ip90 and ACA1 as shown in Fig. 3 herein, the subsequence of the complementary strand being downstream of the first primer in relation to the direction of expression of the first primer, the substantial identity being as defined in claim 1.

- 5 6. A method of strain-specifically detecting the presence of a B. burgdorferi organism of one of the strains B31, ACA1, and Ip90 in animals, including humans, comprising subjecting a specimen from the animal, such as a body fluid, such as blood, serum, cerebrospinal fluid or urine, or a tissue biopsy, or, when the animal is an arthropod, the whole animal, to PCR-DNA analysis using as a primer a DNA sequence which comprises at least 11 nucleotides, the sequence being identical or substantially identical to a subsequence of OspA and OspB DNA from one of the B. burgdorferi strains B31, Ip90 and ACA1 as shown in Fig. 3, which subsequence is different from any subsequence of the two other strains, the difference being such that the sequence will not anneal to DNA from the two other strains, the substantial identity being as defined in claim 1.
- 10 7. A method according to any of the claims 1-6 wherein the primer is labeled.
- 15 8. A method according to claim 7, wherein the primer is labeled with a label selected from radioactive labels, fluorescent dyes, and biotin.
- 20 9. A method according to any of claims 1-6 wherein labeled nucleotide triphosphates are included in the PCR reaction to label the PCR amplification product.
- 25 10. A diagnostic PCR kit for detecting the presence of a B. burgdorferi organism in animals, including humans, substantially independently of the individual strain of the B. burgdorferi, the kit comprising a primer or primer combination as defined in any of claims 1-9.
- 30 11. A DNA fragment comprising a nucleotide sequence as shown in Fig. 3 herein for ACA1, a subsequence thereof which comprises at least 15 nucleotides and which hybridizes to said nucleotide sequence under high stringency hybridization conditions comprising hybridization at 67°C in 2xSSC and final washing at 67°C in 1xSSC or under medium high stringency hybridization conditions comprising hybridization at 67°C in 3xSSC and final washing at 67°C in 1xSSC, or under low stringency conditions such as 6 x SSC and 67°C and subsequent wash at 4 x SSC and 67°C, or a homologue of said nucleotide sequence which encodes a polypeptide identical to or substantially identical to a polypeptide encoded by said nucleotide sequence and which hybridizes to said nucleotide sequence under high or medium high or low stringency hybridization conditions as defined above.
- 35 12. A DNA fragment according to claim 12 comprising a nucleotide sequence starting at nucleotide 37 and terminating at nucleotide 851 of the nucleotide sequence shown in Fig. 3 herein encoding a cell membrane protein from B. burgdorferi, strain ACA1, said protein being designated OspA, or a subsequence or homologue of said nucleotide sequence, the nucleotide numbering being based on the B31 numbering in the manner shown in Fig. 3.
- 40 13. A DNA fragment according to claim 11 comprising a nucleotide sequence starting at nucleotide 867 and terminating at nucleotide 1877 of the nucleotide sequence shown in Fig. 3 herein encoding a cell membrane protein from B. burgdorferi, strain ACA1, said protein being designated OspB, or a subsequence or homologue of said nucleotide sequence, the nucleotide numbering being based on the B31 numbering in the manner shown in Fig. 3.
- 45 14. A DNA fragment comprising a nucleotide sequence as shown in Fig. 3 herein for Ip90, a subsequence thereof which comprises at least 15 nucleotides and which hybridizes to said nucleotide sequence under high stringency hybridization conditions comprising hybridization at 67°C in 2xSSC and final washing at 67°C in 1xSSC or under medium high stringency hybridization conditions comprising hybridization at 67°C in 3xSSC and final washing at 67°C in 1xSSC, or under low stringency conditions such as 6 x SSC and 67°C and subsequent wash at 4 x SSC and 67°C, or a homologue of said nucleotide sequence which encodes a polypeptide identical to or substantially identical to a polypeptide encoded by said nucleotide sequence and which hybridizes to said nucleotide sequence under high or medium high or low stringency hybridization conditions as defined above.
- 50 15. A DNA fragment according to claim 14 comprising a nucleotide sequence starting at nucleotide 37 and
- 55

terminating at nucleotide 851 of the nucleotide sequence shown in Fig. 3 encoding a cell membrane protein from B. burgdorferi, strain ACA1, said protein being designated OspA, or a subsequence or homologue of said nucleotide sequence, the nucleotide numbering being based on the B31 numbering in the manner shown in Fig. 3.

- 5 16. A DNA fragment according to claim 14 comprising a nucleotide sequence starting at nucleotide 867 and terminating at nucleotide 1877 of the nucleotide sequence shown in Fig. 3 encoding a cell membrane protein from B. burgdorferi, strain ACA1, said protein being designated OspB, or a subsequence or homologue of said nucleotide sequence, the nucleotide numbering being based on the B31 numbering in the manner shown in Fig. 3.
- 10 17. A DNA fragment according to any of claims 11-16 which comprises at least 15 nucleotides and which encodes a polypeptide which is capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi.
- 15 18. A substantially pure polypeptide encoded by a DNA fragment according to any of claims 1-17, the polypeptide being substantially free of naturally co-occurring peptides.
- 20 19. A substantially pure polypeptide comprising an amino acid sequence as shown for ACA1 or Ip90 in Fig. 5, an antigenic sub-sequence of said amino acid sequence comprising at least one epitope capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi, or a polypeptide having a homology of at least about 70% with said amino acid sequence.
- 25 20. A substantially pure polypeptide according to claim 19, which is the amino acid sequence OspA as shown for ACA1 in Fig. 5, an antigenic sub-sequence of said amino acid sequence comprising at least one epitope capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi, or a polypeptide having a homology of at least about 70% with said amino acid sequence.
- 30 21. A substantially pure polypeptide according to claim 19, which is the amino acid sequence OspB as shown for ACA1 in Fig. 5, an antigenic sub-sequence of said amino acid sequence comprising at least one epitope capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi, or a polypeptide having a homology of at least about 70% with said amino acid sequence.
- 35 22. A substantially pure polypeptide according to claim 19, which is the amino acid sequence OspA as shown for Ip90 in Fig. 5, an antigenic sub-sequence of said amino acid sequence comprising at least one epitope capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi, or a polypeptide having a homology of at least about 70% with said amino acid sequence.
- 40 23. A substantially pure polypeptide according to claim 19, which is the amino acid sequence OspB as shown for Ip90 in Fig. 5, an antigenic sub-sequence of said amino acid sequence comprising at least one epitope capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi, or a polypeptide having a homology of at least about 70% with said amino acid sequence.
- 45 24. An immunogenic polypeptide fragment comprising a sequence selected from

45

50

55

LVSKEKNKDGKYDL,  
LVSKEKDKDGKYSL,

5

KGTSCKNNGSGV,  
KGTSCKTNGSGV,  
KGTSCKNNGSGT,

10

LEGVKADKSKVKL,  
LEGTKDDKSKAKL,  
LEGEKTDKSKAKL,

15

KKVTSKDKSSTEEK,  
RKVSSKDKTSTDEM,  
KKVTLKDKSSTEEK,

20

KKTKDLVFTKEN,  
KKTTQLVFTKQD,  
RKTKNLVFTKED,

25

QYDSNGTKLEGS,  
KYDSAGTNLEGT,  
KYDSAGTNLEGK,

30

AVEITKLDEIKNALK,  
AVEIKTLDELKNALK,  
AVEITTLKELKDALK,

35

40

45

50

55

5

DLNLEDSSKKSHQNAK,  
DQEI INSDNTPKDSKK,  
DQDVEDLKKDQKDDSK,

10

KIFVSKEKNSSGK,  
KIFVSKEKNSAGK,  
EIFISKEKNEDDK,

15

KPDKSKVKLTVSAD,  
KADKTKVAMTIADD,  
KADKSKVTMLVSDD,

20

KKTGKWEDSTSTL,  
KKTATWNETTNTL,  
KKTAVWNDTSSTL,

25

KNLSELKNALK,  
KDLAALKAALK,  
KDLEALKAALK,

30

and polypeptide fragments comprising sequences which are at least 70% homologous with any of the above sequences and capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi, said polypeptide fragment or fragments being smaller in size than naturally occurring OspA or OspB.

35

25. An antigenic composition comprising, as an antigenic component, a polypeptide encoded by the DNA fragment according to any of claims 11-17 or a polypeptide according to any of claims 18-24.

40

26. An antigenic composition according to claim 25 comprising a combination of polypeptides encoded from at least two of the OspA genes from B31, ACA1, and Ip90.

27. An antigenic composition according to claim 25 comprising a combination of polypeptides encoded from at least two of the OspB genes from B31, ACA1, and Ip90.

45

28. An antigenic composition comprising a combination of at least two polypeptides selected from the group consisting of a polypeptide encoded from a DNA fragment according to claim 11, a polypeptide encoded from a DNA fragment according to claim 14, and a polypeptide encoded by a DNA fragment of OspA or OspB gene from B. burgdorferi, strain B31.

50

29. An antigenic composition according to claim 25 comprising a polypeptide capable of eliciting an immune response against B. burgdorferi of any of the strains B31, ACA1, and Ip90.

30. An antigenic composition according to claim 25 wherein the polypeptide comprises a polypeptide fragment selected from

55

LVSKEKNKDGKYDL,  
LVSKEKDKDGKYSL,

5

KGTSCKNNGSGV,  
KGTSCKTNGSGV, and  
KGTSCKNNGSGT,

10

and polypeptide fragments comprising sequences which are at least 70% homologous with any of the above sequences and capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi of any of the strains B31, ACA1, and Ip90, said polypeptide fragment or fragments being smaller in size than naturally occurring OspA or OspB.

15

31. An antigenic composition according to claim 25 which contains a combination of polypeptides comprising at least one polypeptide fragment selected from

20

LVSKEKNKDGKYDL,  
KGTSCKNNGSGV,  
LEGVKADKSKVKL,  
KKVTSKDKSSTEEK,  
KKTCDLVFTKEN,  
QYDSNGTKLEGS,  
AVEITKLDEIKNALK,  
DLNLEDSSKKSHQNAK,  
KIFVSKEKNSSGK,  
KPDKSKVKLTVSAD,  
KKTGKWEDSTSTL,  
KNLSELKNALK

25

30

35

40

at least one polypeptide fragment selected from

45

50

55

5 LVSKEKDKDGKYSL,  
 KGTSDKTNNGSGV,  
 LEGTKDDKSKAKL,  
 RKVSSKDKTSTDEM,  
 KKTQTQLVFTKQD,  
 KYDSAGTNLEGT,  
 10 AVEIKTLDELKNALK,  
 DQEIINSNTPKDSKK,  
 KIFVSKEKNSAGK,  
 15 KADKTKVAMTIADD,  
 KKTATWNETTNTL,  
 KDLAALKAALK,

20 and at least one polypeptide fragment selected from

KGTSDKNNGSGT,  
 LEGEKTDKSKAKL,  
 25 KKVTLKDKSSTEEK,  
 RKTKNLVFTKED,  
 KYDSAGTNLEGG,  
 30 AVEITTLKELKDALK,  
 DQDVEDLKKDQKDDSK,  
 EIFISKEKNEDDK,

35 KADKSKVTMLVSDD,  
 KKTAVWNDTSSTL,  
 40 KDLEALKAALK,

45 and polypeptide fragments comprising sequences which are at least 70% homologous with any of the above sequences, the combination being capable of interacting with immunocompetent cells to elicit an immune response against B. burgdorferi of any of the strains B31, ACA1, and Ip90, said polypeptide fragment or fragments being smaller in size than naturally occurring OspA or OspB said polypeptide fragments being smaller in size than naturally occurring OspA or OspB.

- 50 32. A vaccine for immunizing mammals, including humans, against Lyme disease, the vaccine comprising an antigenic composition according to any of claims 25-31 and an immunogenically acceptable carrier or vehicle.
33. A vaccine according to claim 32, wherein the carrier or vehicle is selected from macromolecular carriers such as a polymer, e.g. a polysaccharide or a polypeptide.
- 55 34. A vaccine according to claim 32 or 33, which additionally comprises an adjuvant.
35. A vaccine according to claim 34, wherein the adjuvant is selected from the group consisting of Freund's

complete or incomplete adjuvant, aluminum hydroxide, a saponin, a muramyl dipeptide, and an oil.

36. A vaccine according to any of claims 32-35 which additionally comprises a T-cell epitope peptide.

5

10

15

20

25

30

35

40

45

50

55



Fig. 1

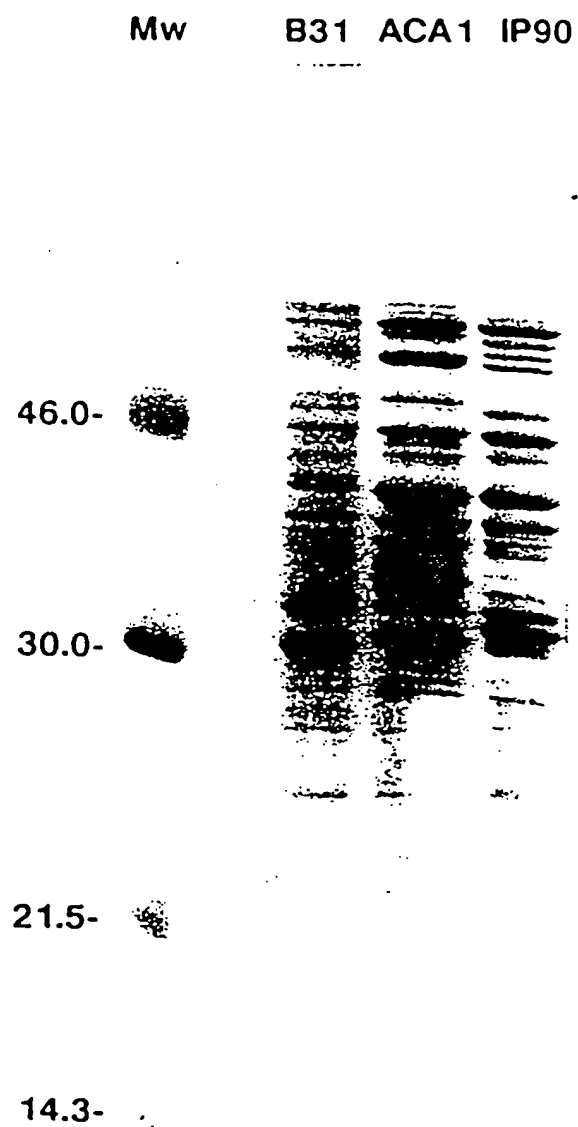


Fig. 2a

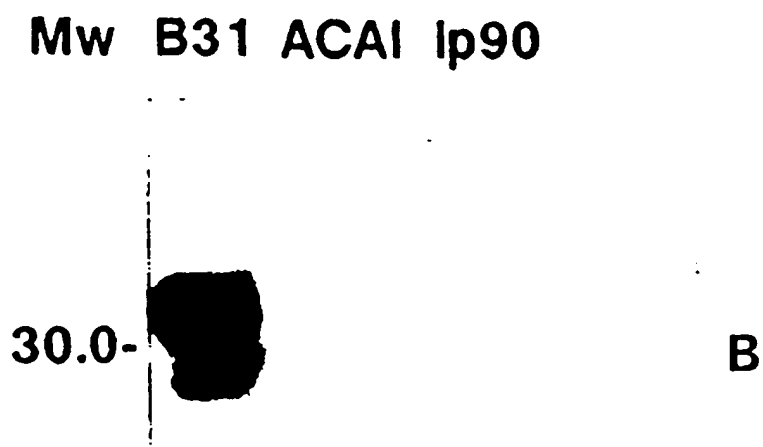
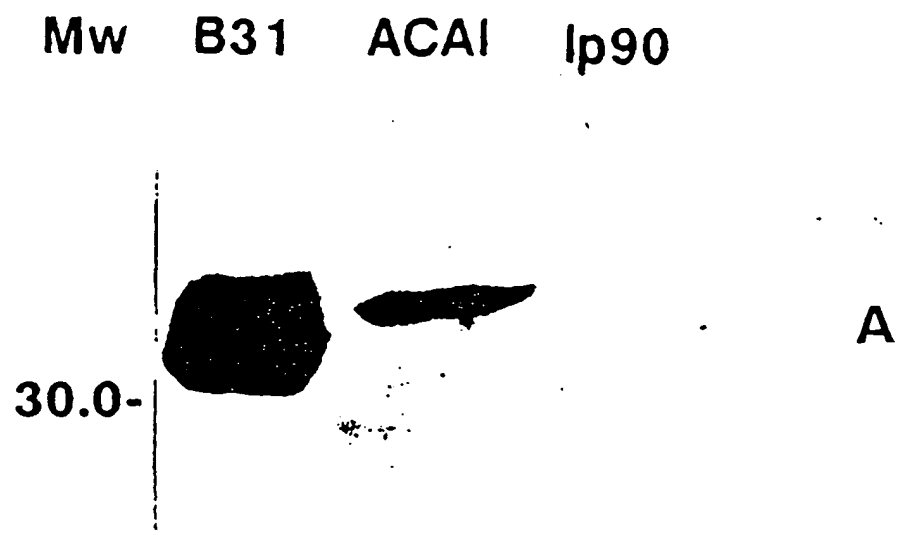
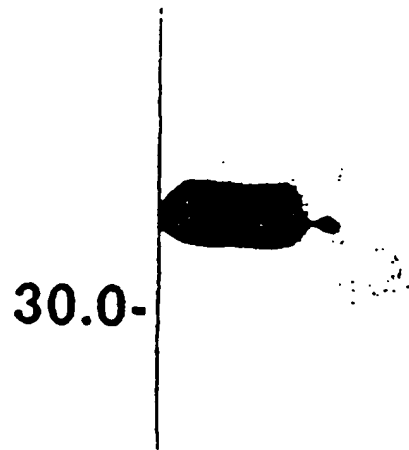


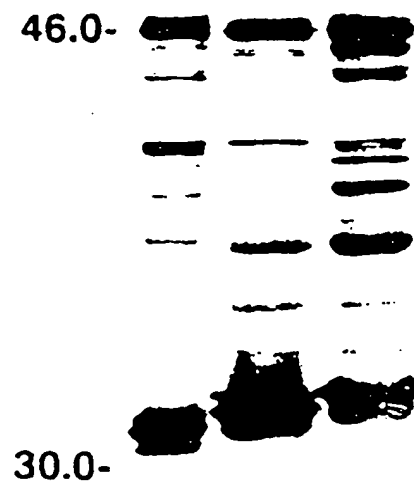
Fig. 2 b

Mw B31 ACAI Ip90



C

Mw B31 ACAI Ip90



D

**Fig. 3a**

36

**Fig. 3b**

920  
 960  
 980  
 990  
 1000  
 1040  
 1080  
 1120  
 1160  
 1200  
 1240  
 1280  
 1320  
 1360  
 1400  
 1440  
 1480  
 1520  
 1560

B31  
 ACAI  
 Ip90

B31  
 ACAI  
 Ip90

B31  
 ACAI  
 Ip90

B31  
 ACAI  
 Ip90

B31  
 ACAI  
 Ip90

B31  
 ACAI  
 Ip90

B31  
 ACAI  
 Ip90

Fig. 3c

[illegible]

Fig. 4





**Fig. 5a**

[illegible]

	60	90
B31	ATVDKLELKGTS	DKNNGSVLEGVKADSKVKLTISDDLGGQTLEVFREDGKTLV
N40	-----	-----
ZS7	-----	-----
ACAI	-----I-----D-----T-D-----A-----A-----SK-F-L-----	-----
Ip90	-----T-----E-T-----A-----AE-----SK-F-I-----	-----

[illegible]

	180	210
B31	VLEGTLTAE	KTTLVVKEGTVTL
N40	-----	SKNISKSGEVSVELNDTDSSAATKKTAAWNSG
ZS7	-----	-----
ACAI	T--KVAND	-V--E-----E-A--T-A--NTTQ--G-D-K
IP90	A---A-DG--	-K-T---V---H-N--IT---S-TTQ---GT-D-K

B31		240	270
N40	TSTLTITVNSKRTDLPFTKEN	ITVQQYDSNGTKLEGS	AVEITKLDEIKNALK
ZS7	-----	-----	-----
ACAI	-----	-----	-----
Ip90	S--TQ--QD--K--A-N-T--KT--L--		
	S-R-N---D---K---A-N-K---T-K-L-D---		



European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number

EP 92 61 0073  
Page 1

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	RESEARCH IN MICROBIOLOGY vol. 142, no. 5, 1991, PARIS, FRANCE pages 565 - 572 M. DEBUE ET AL. 'Detection of Borrelia burgdorferi in biological samples using the polymerase chain reaction assay' * page 566, left column, line 4 - line 18 * * page 567, left column, line 20 - page 568, right column, line 12; table 2 * ---	1	C12Q1/68 C12N15/31 C07K13/00 C07K7/00 A61K39/02
X	PROTEIN EXPRESSION AND PURIFICATION vol. 1, 1990, NEW YORK, USA pages 159 - 168 J.J. DUNN ET AL. 'Outer surface protein A (OspA) from the lyme disease spirochete, Borrelia burgdorferi: High level expression and purification of a soluble recombinant form of OspA' * see the whole document, especially abstract and "discussion" * ---	19-21	
X	MOLECULAR MICROBIOLOGY vol. 3, no. 4, April 1989, NEW YORK, USA pages 479 - 486 S. BERGSTRÖM ET AL. 'Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the lyme disease spirochaete Borrelia burgdorferi' * the whole document * --- -/--	15-24	TECHNICAL FIELDS SEARCHED (Int. Cl.5)  C12Q C07K
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 21 JANUARY 1993	Examiner LUZZATTO E.R.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure F : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document			

EPO FORM 1503 (01.82) (P0601)



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

EP 92 61 0073

Page 2

DOCUMENTS CONSIDERED TO BE RELEVANT				
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. CL.5)	
D,A	JOURNAL OF CLINICAL MICROBIOLOGY vol. 29, no. 3, March 1991, WASHINGTON, DC, USA pages 524 - 532 P.A. ROSA ET AL. 'Polymerase chain reaction analyses identify two distinct classes of <i>Borrelia burgdorferi</i> ' * abstract * * figure 2 * * "discussion" *	1		
A	JOURNAL OF CLINICAL MICROBIOLOGY vol. 28, no. 3, March 1990, WASHINGTON, DC, USA pages 566 - 572 D.H. PERSING ET AL. 'Detection of <i>Borrelia</i> <i>burgdorferi</i> infection in <i>Ixodes dammini</i> Ticks with the polymerase chain reaction' * the whole document *	1		
A	WO-A-9 004 411 (SYMBICOM AKTIEBOLAG) 3 May 1990 * page 15, line 14 - line 25; claims 24,27,68-72 *	1,12-18		TECHNICAL FIELDS SEARCHED (Int. CL.5)
A	EP-A-0 418 827 (MAX-PLANCK GESELLSCHAFT) 27 March 1991 * page 2, line 55 - page 3, line 18; claims *	19-32		
P,X	WO-A-9 200 055 (YALE UNIVERSITY) 9 January 1992  * page 10, line 4 - page 17, line 5 * * page 24, line 7 - line 24 *	13-26, 30,31, 33-37		
The present search report has been drawn up for all claims				
Place of search THE HAGUE		Date of completion of the search 21 JANUARY 1993	Examiner LUZZATTO E.R.	
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons * : member of the same patent family, corresponding document</p>				

EPD FORM 1500 03.92 (P0601)

